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(54) Title: USE OF HEPARINS FOR THE TREATMENT OF INFLAMMATORY OR IMMUNOLOGICAL DISEASES

(57) Abstract

A pharmaceutical for the treatment of inflammatory or immunological diseases and a method of treatment of these diseases. The invention is characterized in that the pharmaceutical comprises heparins, heparinoids, proteoglycans or low-molecular-weight heparins or a mixture thereof or a combination of low-molecular-weight heparins and Prostavasin.

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USE OF HEPARINS FOR THE TREATMENT OF INFLAMMATORY OR IMMUNOLOGICAL DISEASES

Background of the invention

Field of the invention

The invention is related to a pharmaceutical composition for the treatment of inflammatory and/or immunological diseases and a method for the treatment of said diseases. These diseases can be multiple sclerosis, primary biliary cirrhosis, rheumatism, lupus erythematosus (LE), post-infarct-syndrome, Ghost versus Host reaction (GvH), auto-agressive neuritides (german: Neuritiden), migraine, hyper-IgE-syndrome, Crohn's disease (german: Morbus Crohn), systemic carcinoma diseases and the like. Systemic carcinoma diseases can be melanoma, Burkitt lymphoma, leukaemia, Non-Hotchkin lymphoma and the like.

Description of the prior art

Heparin and heparan sulfate which are known as anticoagulant substances found in the liver and lungs and which can also be produced artificially are normally used for the treatment and prophylaxis of thrombosis. The above-mentioned pharmaceuticals are known at least since 1930. They are especially used for the treatment of hyperlipidaemia, arteriosclerosis and are used during blood transfusion and after operations. The interaction of heparins with lymphocyte adhesion patterns was studied by Ekre et al. (Ekre H.P., Fjellner B. and Hagermark O. (1986) Inhibition of complement dependant experimental inflammation in human skin by different heparin fractions. Int. J. Immunophramacol. 8 (3):277-286; in the following

Ekre et al.1986) concerning the activity of heparin which suppresses the immunoresponse in the human skin permantently at concentrations between 2 and 5 i.U. per kg and day with a dose limitation of 300 i.U. per day. However, it is not known to use these pharmaceuticals for treating inflammatory or immunological diseases.

One of the most known adhesion molecule is the fibrinogen in the tissue-plasminogen-activator-system (TPA). There are also other important systems. These are e.g. fibronectin and lactoferrin which exist in large amounts if the patient has tumors and which are a sign for the decreasing cellular contact inhibition. These proteins are also detectable for chronic inflammations which do not have a tumoric origin and are called acute-phase-proteins (german: Akutphasenproteine). Our own results show that the acute-phase-proteins are increased corresponding to the C-reactive protein and are directly correlated to the erythrocyte sedimentation rate.

Multiple sclerosis (MS) has become a major health problem in industrialized countries. It affects between 50 and 100 individuals out of 100.000. A review of the literature gave evidence that up to now there is no convincing clue what the main cause of this disease could be (Reinherz E.L., Kung P.C., Goldstein G. and Schlossmann S.F. (1979) Separation of functional subsets of human T-cells by monoclonal antibody. Proc.Nat.Acad.Sci.USA 76:4061; in the following: Reinherz et al. 1979). Until recently no causal therapy was avalaible.

Other inflammatory and/or immunological diseases are also a major health problem not only in industrialized but also in countries at the stage of economic take-off and also

those of the third world. These diseases are e.g. primary biliary cirrhosis, rheumatism, lupus erythematosus (LE), post-infarct syndrome, ghost-versus-host reaction (GvH), systemic carcinoma diseases and others.

Various pharmaceuticals are used to treat the above mentioned diseases. However, there does not exist any pharmaceutical which allows a healing of the patients without any side effects. Moreover, the probability of a complete recovery of the patients is low. It is thus an object of the invention to find a pharmaceutical and a method of treatment to cure patients from the above mentioned diseases with few or even no side effects at all.

The high endothelial cells (HEC), oligodendrocytes and Schwann cells of multiple sclerosis (MS) patients carry a hypervariable activated cellular DNA, a so called inter-cellular adhesion molecule cellular DNA (pICAMhec cDNA) which encodes a hypervariable ICAMhec. Activated lymphocytes of the type Ta_1 and $T11_3$ adhere strongly to cells expressing this ICAMhec and from the studies of Ford (Ford W.L. (1978) Possible clues to the mechanism underlying selective migration of lymphocytes from the blood. Symp. Soc. Exp. Biol. 32:359; in the following: Ford 1978) and Woodruff et al. (Woodruff J.J. and Kuttner B.J. (1980) Adherence of lymphocytes to HEV of lymph nodes in vitro Excerpta Medica: Blood cells and vessels walls: functional interactions (Cibal Foundation Symposium) 243-263; in the following Woodruff et. al.1980) we know that adherent lymphocytes migrate into the surrounding tissue. A possible role of cell surface molecules involved in this adhesive interaction has been postulated already for some time by Stoolman et al.

(Stoolman L.M. and Rosen S.D. (1983) Possible role for cell surface carbohydrate binding molecules in lymphocyte recirculation. J. Cell Biol.96:722; in the following: Stoolman et. al.1983). In a routinely performed adhesion assay we observed that lymphocytes preincubated with MS patient serum adhere on HEC.

However, it was not known which type of lymphocytes adhere on HEC and secondly which molecules regulate this adhesion. From the literature published by Reinherz et al.1979, Bach et al. (Bach M.A., Tournier E. et al.(1980) Deficit of suppressor T-cells in active multiple sclerosis. Lancet 2:1221; in the following Bach et al.1980), Traugott et al.(Traugott U., Reinherz E.L. and Raine C.S. (1983) Multiple Sclerosis: distribution of T-cells subsets within active relapsing and chronic progressive diseases. Ann. Neurol 14:445; in the following: Traugott et al.1983) and Weiner et al. (Weiner H.L., Bhan A.K. and Burks J. (1984) Immunohistochemical analysis of the cellular infiltrate in multiple sclerosis lesions. Neurology (NY) 34: Suppl 1:112; in the following Weiner et al.1984) it is known that the adherent lymphocytes could be Ta_1 and $T11_3$ cells.

Objects and Summary of the Invention

It is therefore an object of the invention to provide an improved pharmaceutical composition for the treatment of inflammatory and immunological diseases and a method of treatment of said diseases.

In accordance with these objects the invention provides pharmaceutical compositions comprising heparins, heparinoids, proteoglycans or low-molecular-weight heparins or mixtures therof to be used for the treatment

of the above-mentioned diseases. The present invention furthermore provides a method of treatment of such diseases involving the use of said pharmaceutical compositions.

The pharmaceutical compositions of the invention and the method of treatment make it possible to cure patients from the above-mentioned diseases with only few side effects or even no side effects at all.

The pharmaceuticals according to the invention generally comprise heparins of various molecular weights. Preferably, low-molecular-weight heparin is used. However, heparins of high molecular weight, heparinoids, heparan sulfate as well as proteoglycans can also be used.

The pharmaceutical can be applied directly to the mucosa or it can be injected subcutaneously or intravenously. It should be noted that the pharmaceuticals should not be applied to the eye because there a toxic reaction could occur. For a usage of the pharmaceutical nasally a spray seems to be suitable so that the patient is able to treat himself easily with the pharmaceutical. Swallowing pills is another possibility for an easy treatment of the patient himself because the dosage can be controlled very accurately. However, an injection is also suitable.

The dosages for the treatment depends on the disease itself and its strength and the pharmaceutical which is used. However, it turned out that dosages of up to 40 i.U per kg and day are suitable to effect MS-patients and also not more than 40 i.U. per kg and day should be suitable to effect patients with the other mentioned diseases. It is

most suitable to use dosages between 1 and 5 i.U. for all mentioned diseases.

If the patient will be treated with one of the mentioned pharmaceuticals it is important to mention that the international units are not always defined the same manner. One should always calculate the correct equivalent molar concentration to e.g. Fragmin-D. If e.g. heparin is used a factor of ca. 30 has to be taken to receive the correct amount of this pharmaceutical. If other heparinoids or heparan sulfate are used the correct equivalent molar concentration must also be calculated. The used dosage depends on the concentration of the pharmaceutical in the plasma (german: Plasmaspiegel) which should be more than 0,5 i.U./ml for LWHM's.

Most suitable for diseases like MS, rheumatism, primary biliary cirrhosis, migraine, hyper-IgE-syndrome and neuritides are pharmaceuticals as Fragmin and Fragmin-D. It turned out that for diseases like GvH and systemic carcinoma e.g. leukaemia or lymphoma Reviparin is a preferred pharmaceutical.

If Fragmin is used a dosage between 1-40 i.U. antifactor Xa per kg and day which means an amount of more than 0,5 i.U./ml in the plasma (german: Plasmaspiegel) should be achieved.

The present invention is based on observations obtained in in vitro studies on the influence of heparin on the adhesive interaction between HEC cells and peripheral blood lymphocyte subtypes.

Between 1985 and 1991 we took blood samples from 1216 patients and we conducted adhesion assays on cultivated high endothelial cells (HEC). We observed reproducible pathognomonic lymphocyte adhesion of two types of peripheral blood lymphocytes, one bearing the T-cell-specific activation antigen Ta_1 and the other activation antigen is the so called $T11_3$ lymphocytes to HECs. Therefore, we started analyzing the Intercellular Adhesion Molecule (ICAM) on HEC, oligodendrocytes, Schwann cells and lymphocytes as well as the Lymphocyte Function Antigen (LFA) on Ta_1 and $T11_3$ lymphocytes of MS patients by means of protein structure analysis. Furthermore, in situ hybridization to the ICAM's and LFA genes in lymphocytes and high endothelial cells of MS patients was carried out.

It was found that MS patients express a pathognomonic hypervariable cellular DNA which encodes a hypervariable ICAMhec on HEC, oligodendrocytes and Schwanncells in 95% of the cases. The metabolized degradation products of this ICAMhec product leads to the activation of T-cells, i.e. the expression of Ta_1 and $T11_3$ antigens on lymphocytes. The molecular structure of this ICAMhec shows a 75% homology to heparansulfateproteoglycane. Adding heparin to Ta_1- and $T11_3$-positive lymphocytes inhibited the pathognomonic adhesion patterns of MS patients lymphocytes. The evaluation of these in vitro studies led to the concept of the pharmaceutical compositions and the method of treatment of the present invention.

The results of extensive studies show that the effect of inhibition of coagulation of the pharmaceutical is caused by a blocking or modelling of adhesion molecules including those expressed on thrombocytes. This effect prevents the

thrombocytes or other blood cells from aggregating or from adhering to an injured vascular wall.

Moreover, the viscosity of the blood cells is altered by medication with the pharmaceutical in such a manner that they can increase their length up to 7 times over their physiological length.

The results show that the pharmaceutical has a direct effect on the LFA-receptors and the ICAM-system because it has the function of a modulating receptor or ligand for both systems. Fibronectin, lactoferrin, fibrinogen and the whole TPA and others belong also to this system. Moreover, the suppression of transplantation and GvH reactions can be explained by the effect on the LFA and the ICAM.
The diseases to be treated can be multiple sclerosis, GvH, primary biliary cirrhosis, post-infarct-syndrome, LE, rheumatism or systemic carcinoma. The dosage of this pharmaceutical should be up to 40 i.U. (international units) per kg and day. However, a dosage between 1 and 5 i.U. per kg and day is preferred.

The NMR controlled reduction of sclerotic patches with LMWH for example Fragmin-D is significant and the number of negative side effects compared to other standard drugs such as dexamethason or azathioprine is negligible. The only observed side effects were a distinct sleepiness, nausea, and ophthalmic migraine. The sleepiness persisted usually during the whole treatment period. Therefore, most patients received their medication in the evening. Nausea and migraine disappeared within a fortnight. A combination of Prostavasin with LWHM leads to an improved biological availability. Thus, Prostavasin is used to improve local blood flow. This is another new therapeutic regime with

rather encouraging results. Prostavasin is a prostaglandin. The substance is Alprostadil. However, it has to be proven which other possible indications for this drug are.

We found that low-molecular-weight heparins in vitro as well as in vivo have significant better antiflammatory and antiactivation potentials in MS than the medication tested in this study ($p = 0{,}002$; $n = 1216$). There was less positive influence by dexamethason or azathioprine compared to heparin (LMWH).

Other objects and advantages of the present invention and some more results of the extensive studies will become apparent from the following detailed description of the invention when considered in conjunction with the accompanying drawings.

Brief Description of the Drawings

Fig. 1: The results of the analysis of peripheral blood mononuclear cells with anti-Ta_1, anti-$T11_3$ and control ascites in a patient with progressive MS and a healthy control are shown. The flowcytometric analysis was performed with a linear scale of fluorescence intensitiy.

Fig. 2: The Immunoprecipitation of ICAM from ^{125}J-labelled cells was demonstrated by the use of the monoclonal antibody 84-H10.

- a: Regulation of ICAM synthesis in HEC by cytokines;
- b: Regulation of ICAM synthesis in oligodendrocytes by cytokines;

Fig. 3: One can observe the expression and structure of the pICAMhec gene shown by method
a: RNA blot hybridization and
b: genomic DNA blot hybridization.

Fig. 4: Reduction of sclerotic patches and symptom improvement after longterm treatment of MS-patients with Fragmin-D, dexamethasone, azathioprine or a control substance.

Fig. 5: Significant therapeutic improvement in 73 acute MS cases after treatment with LMWH compared to treatment with dexamethasone or azathioprine ($p = 0{,}002$; $n = 73$).

Fig. 6: Nucleotide sequence of the cellular DNA clone pICAMhec (a), and the associated protein sequence (b). The nucleotide numbering goes from left to right, the aminoacid sequence from right to left.

Fig. 7: Typical alteration of the adhesion rate in a case of MS. The last attack was 15 years ago.

Fig. 8: Typical picture of an acute MS attack. Upon serial dilution more than 10.000 adherent lymphocytes per HEC were observed in the adhesion assay.

HEC were cultivated according to v. Arnim (1987, German Patent No. DE 3536955). $5x10^6$ HEC per ml were cultivated on PRIMARIA-multiwell plates (Becton & Dickinson, Heidelberg) with culture medium RPMI-1640 (Gibco, Karlsruhe) containing 16% Hyclone defined Fetal Calf Serum, 200mM L-glutamine, 20 µg/ml Endothelial Cell Growth Supplement (ECGS; Sigma), 20 µg/ml Multiple Stimulating Activity

(MSA; Sigma), 10 µg/ml Heparin (H-3125, Sigma) and 15 µg/ml Insulin (Sigma). After a 10 day cultivation period the HEC-monolayer was confluent and therefore the cells were fixed for 10 min at 4°C in 0,2Mol L-lysine (Gibco, Karlsruhe) and deepfrozen in liquid nitrogen.

The blood samples were collected by iv puncture. The lymphocytes and the serum of each sample were extracted by centrifugation in Lymphoprep-tubes (Becton & Dickinson, Heidelberg). Thereafter serum and lymphocytes were deepfrozen in liquid nitrogen.

Anti-Ta_1 immunoprecipitates a single major 105 kd band. Unlike antibodies to the interleukine-2 receptor, anti-Ta_1 does not inhibit T cell proliferative responses to antigen or interleukine containing medium. Anti-$T11_3$ reacts with a unique epitope that becomes expressed on T cells after activation. The weight of the pICAMhec cDNA encoded protein varies between 100 and 125 kd. Concerning also the 105 kd band immunoprecipitated by anti-Ta1 and LFA-1 being a glycoprotein of a weight also varying between 100 and 125 kd this leaves no other conclusion then ICAM being the ligand of LFA-1-carrying Ta_1-cells (Stoolman L.M. (1989) Adhesion molecules controlling lymphocyte migration: Cell 56:907-910; in the following: Stoolman 1989). Taking all this into account ICAM expressing cells play a major role in the induction of the pathophysiologic response in MS. Gesner et al. (Gesner B.M. and Ginsburg V. (1964) Effect of glycosidases on the fate of transfused lymphocytes. Proc. Natl. Acad. Sci. USA 52:750; in the following: Gesner et al.1964) , Ford 1978, Curtis (Curtis A.S.G. (1974) The specific control of cell positioning. Arch. Biol. 32:105; in the following: Curtis 1974) and Aklyama (Aklyama S.K. (1981) The structure of fibronectin and

its role in cellular adhesion. J. Supramolecular structure and cellular Biochem. 16:345; in the following Aklyama 1981) postulated that cells involved in lymphocyte adhesion and migration must (1) produce, release and transport recognition signal; (2) receive signals; (3) activate an effector system in the receiving cell; and (4) induce a specific answer in the receiving cell via the effector system. In case of the adhesion of Ta_1 and $T11_3$ cells on ICAMhec expressing cells. Stoolman et al.1983 and Holzmann et al. (1989) have demonstrated the specificity of adhesion, migration and cellular interaction and its physiologic versus pathophysiologic relevance. The LFA-1 is a member of the integrin family of cell surface receptors (Stoolmann 1989). The tripeptide motif Arg-Gly-Asp (RGD) is a common feature of the ligands for this family and is required for ligand receptor interaction.

But ICAM contains no RGD motifs bearing instead a single RGE sequence at position 152 (see Fig.4). Nevertheless there is a striking similarity between ICAMhec, NCAM and between heparansulfatproteoglykane and LFA-1. This similarity is particularly interesting as it brings together surface mucopolysaccharids, lymphoid and neuronal adhesion molecules.

According to Reinherz et al.1979, Bach et al.1980, Weiner et al.1984 and Traugott et al.1983 multiple sclerosis (MS) patients carry Ta_1 and $T11_3$ lymphocytes. Traugotts laboratory gave us Anti-Ta_1 and Anti-$T11_3$. All monoclonal antibodies were used in antibody excess at a dilution of 1:500 except for anti-T3, which was used at a dilution of 1:250. Cytofluorographic analysis of cell populations were performed by means of indirect

immunofluorescence with fluoresceinisothiocyanate-conjugated (FITC) goat antirat antibodies (Wellcome) on a flow cytometer using a linear scale. Background fluorescence activity was determined by control ascites fluid obtained from rats immunized with a nonsecreting hybridoma.

For the adhesion assays lymphocytes from healthy controls (no known immunologic, allergic or infectious disease) were incubated in patient serum for 60min at 37°C, centrifuged with 300g, washed in phosphate buffered saline (PBS), centrifuged once more and resuspended in RPMI-1640 containing 10mM magnesium. Thereafter the fixed HEC-monolayer was incubated with the resuspended lymphocytes for 60min at 37°C. After 60min the incubated monolayer was washed three times with PBS, stained with acridine orange and observed under the microscope. The number of adherent lymphocytes per HEC was documented by photography (Film Agfachrome professional ISO 200).

Peripheral blood mononuclear cells of a patient with progressive MS and of a healthy control individual were analyzed with anti-Ta_1, anti-$T11_3$ and control ascites by flowcytometric analysis with a linear scale of fluorescence intensity (see Fig. 1). Ta_1 lymphocytes in both T4 and T8 subsets from a patient with MS. Subsets were prepared by complement-mediated lysis of reciprocal populations and stained with anti-T3, anti-T4, anti-T8, anti-Ta_1 and control ascites. Cytofluorographic analysis using a log scale was then performed. The T8 subset shows staining with anti-T3, anti-T8 and anti-Ta_1, and no staining with anti-T4. The reciprocal T4 subset also stains with anti-Ta_1. Anti-T3 reacts with virtually all peripheral blood T cells. Anti-Ta_1 immunoprecipitates a

single major 105 kd band. Unlike antibodies to the interleukine-2 receptor, anti-Ta_1 does not inhibit T cell proliferative responses to antigen or interleukine containing medium. Anti-$T11_3$ reacts with a unique epitope that becomes expressed on T cells after activation. The percentage of activated antigen positive T cells was calculated by dividing the number of activated antigen positive T cells by the total number of T3-positive cells. The average percentage of T3-positive mononuclear cells was significantly different in the different groups.

To immunoprecipitate the Inter Cellular Adhesion Molecule (ICAM) on ^{125}J-labelled HEC, oligodendrocytes, and Schwann cells we used the commercially available monoclonal antibody 84-H10 (Becton & Dickinson, Heidelberg). A transfection with plCAMhec was done according to the protocols of Berger et al. (Berger S.L. and Kimmel A.R. (1987) Guide to molecular Cloning techniques Academic Press Inc. Vol 152:1-812; in the following: Berger et al.1987) and Glover (Glover D.M. (1987) DNA cloning a practical approach. IRL Press Vol 1-3; ed.: D.M. Glover; in the following: Glover 1987) episomal DNA was extracted from washed HEC. The HEC, oligodendrocytes, Schwann cells were set to $5x10^5$ cells per ml. Thereafter, the cells were incubated for 48h with the following test reagents; 50ng/ml 12/0/tetradekanoylphorbol-13-acetate (TDA, Sigma), 100µ/ml gamma-interferon, 200µ/ml TNF, 10µ/ml interleukine-1-beta. For the function analysis the HEC, oligodendrocytes and Schwann cells were transfected with the control vector PE-H3M. We then conducted a lymphocyte adhesion assay. Therefore HEC, oligodendrocytes and Schwann-cells were incubated for 1h at 37°C in PBS containing 12,5% defined fetal calf serum (Hyclone) and 1µg/ml of the standard

antibody 84-H10. Once in each preparation the antibody was washed of to avoid lymphocyte binding by the antibody excess. In all the other cases the antibody excess was necessary to avoid lymphocyte migration through the cells.

The immunoprecipitation of ICAM from ^{125}J-labelled cells was demonstrated by the use of the monoclonal antibody 84-H10 (Fig. 2)
a: Regulation of ICAM synthesis in HEC by cytokines; gamma-interferon-induced without antibody (lane 1), with antibody (lane 2); tumor necrosis factor (TNF)-induced without antibody (lane 3) with antibody (lane 4)
b: Regulation of ICAM synthesis in oligodendrocytes by cytokines; gamma-interferon-induced without antibody (lane 1), with antibody (lane 2); TNF-induced without antibody (lane 3), with antibody (lane 4); interleukine-1β-induced without antibody (lane 5), with antibody (lane 6).

In Fig. 3 one can observe the expression and structure of the pICAMhec gene shown by the method of
a: RNA blot hybridization. Total RNA (24 µg) was denatured in formaldehyde, electrophoresed, transfered to nylon membranes and hybridized with ICAM-1 cDNA.
Lane 1 uninduced HEC; lane 2 TDA induced; lane 3 gamma IFN induced; lane 4 IL-1β induced; lane 5 TNF induced; lane 6 glomerular endothelium; lane 7 oligodendrocytes from an MS patient; lane 8 Ta_1; lane 9 $T11_3$; lane 10 lymphokine activated killer cells. The RNA blot analysis revealed a 3,2 kb and a 1,9 kb species in HEC stimulated with TDA, gamma-interferon, TNF, and interleukine-1β. It was not found in uninduced cells. The 1,9 kb band is blocked by 18S rRNA which is present as a contamination in poly-A^+ selected RNA and is therefore considered unspecific.

The results lead to the conclusion that the expression of ICAM is regulated by inflammatory cytokines on the transcription level.

b: genomic DNA blot hybridization (pICAMhec cDNA from high endothelial cells from MS patients): lane 1: EcoRI; lane 2: BamHI; lane 3: HindIII endonuclease from Hemophilus influenza; lane 4: DraI endonuclease.

For the DNA-hybridization human chromosomal placenta DNA (24µg) was digested with restriction enzymes, separated electrophoretically and transfered to a nylon membrane. Then it was hybridized with the cellular pICAMhec-cDNA clone. For the RNA blot hybridization we used the protocol from Berger et al.(1987) and Glover (1987); 24µg cellular RNA was denatured in formaldehyde, separated electrophoretically and transferred to a nylone membrane. Thereafter the RNA was hybridized with cellular DNA of the clone plCAMhec-cDNA. A cellular DNA library was constructed by extracting RNA stimulated with 12/0/tetradecanoylphorbol-13-acetate (TDA; Sigma) from MS patients' lymphocytes, HEC, oligodendrocytes and Schwann cells. This library was transfected into HEC. HEC expressing the pICAMhec were identified by the antibody 84-H10. From these cells the episomal DNA was extracted to isolate the clone pICAMhec-cDNA. The sequencing was conducted via the dideoxy-chain-termination using a combination of subclones and specific oligonucleotides and the align program of the Protein Identification Ressours. The T cells for the tests werde obtained by culture of peripheral blood lymphoyctes with phytohemagglutinin for 48h.

Synthesis of a cDNA library:
RNase H from Escherichia coli is used to nick the RNA in the hybrids, leaving only small RNA primers with free 3'-OH groups attached to the cDNA. These 3'-OH groups can subsequently be used by DNA polymerase I to synthesize efficiently a second strand all along the length of the first strand (original cDNA). Double stranded oligonucleotides called BamHI-linkers are used to add complementary ends to blunt-ended double helical DNA. The linkers are first ligated to both ends of the given DNA. Then the product is treated with BamHI-endonuclease. HindIII endonuclease is from Hemophilus influenzae. The rest of the synthesis of the cDNA library is in accordance to Kimmel et al. (Kimmel, A.R. and Berger, S.L.: Preparation of cDNA and Generation of cDNA Libraries: Overview, Methods in Enzymology, 1987, 152:307).

The here described transcript pICAMhec which encodes a protein of a relative molecular weight of 110 kd was discovered in T cells induced by MS. Therefore, we established the homogeneity of the pICAMhec transcript and the activated pICAMhec-cDNA. The blot-hybridization of the pICAMhec transcript with the placental genomic DNA revealed a pattern indicating a single copy gen. To establish the pathognomic role of the pICAMhec encoded protein HEC, oligodendrocytes and Schwann cells expressing pICAMhec were studied according to their ability to bind Ta_1 and $T11_3$ cells. Within 60 min Ta_1 and $T11_3$ cells adhered strongly to HEC, oligodendrocytes and Schwann cells expressing pICAMhec while the lymphocytes did not adhere to cells with a related transfection.

The sequencing of the cellular DNA clone pICAMhec and the associated protein sequence is shown in Fig. 6. The

nucleotide numbering goes from left to right, the aminoacid sequence from right to left. The RGD-motive at position 152 is underlined, the potential N-dependent point of glycosilation is marked by "-CHO-". The transmembrane domain is stained by "-TM-". The aminoacid sequence is numbered from the projected separation point of the signal peptide. Remaining cysteine units are circled. The pICAMhec-cDNA clone is build out of 1846 ± 185 nucleotides. The computer analysis of 40 different pICAMhec clones from HEC, oligodendrocytes, and Schwann cells compared to 40 clones isolated from lymphocytes showed that under physiologic conditions the intercellular sequence variability is less than 10%. Under pathophysiological conditions the intercellular sequence variability is also less than 10% but the interpatient variability is more than 25%. As ICAM belongs to the family of the immunoglobulins we established another computer analysis and found out that the pICAMhec-cDNA clone expresses a constant region with 1600 ± 160 nucleotides and a hypervariable region with 246 ± 25 nucleotides. The constant region has a sequence variability of less than 5% while the hypervariable region has a variability of more than 60%.

The predicted protein sequence has the typical characteristics of a transmembrane protein, which includes a potential signal sequence, a possible separation point a glycine-25 and asparagine-26, and a singular 25 unit transmembrane domain which terminates in a cytoplasmatic domain of high potential. The extracellular domain includes 7 potentially N-linked glycosylation points. At least this could explain the weight difference between the deglycosylated precursor (60kd) and the endproduct (100-125kd). The specific use of these glycosylation points

could also explain the heterogenic molecular mass of the plCAMhec product i.e. ICAMhec. A search of a laboratory database containing recently published surface proteins, however, did reveal a surprising and significant similarity between ICAMhec, the neuronal cell adhesion molecule (NCAM). The optimal alignment score obtained, using the National Biomedical Research Foundation (NBRF) Align Programm is eight standard deviation above the mean score obtained from 500 random permutations of the sequence. Using a database of known immunoglobulin related sequences it has been shown that ICAMhec may be divided into five domains (28-112, 115-206, 217-310, 312-391, and 399-477) each of which shows significant similarity with members of the immunoglobulin. For example domain I is similar to CD3, whereas domain III and IV align to domains of myelin associated glycoprotein (Holzmann B., McIntyre B.W. and Weissman I.L., Cell 1989 56:37-46) and a cinoembryonic antigen (Stoolman 1989). All five domains of ICAMhec align with NCAM.

Example 1:
Studies on MS patients were carried out with the assumption that heparins might be potentive immuno-suppressors in human at concentrations of 2-5 i.U. per kg and day. Therefore, a placebo controlled doubleblind crossover study with a Low-Molecular-Weight-Heparin (LMWH) (Fragmin-D, Kabi, Erlangen) was performed. Fragmin-D was chosen because the halflife time is 5 times longer than that of other heparins and the dilution row revealed that its antiinflammatory activity is 30 times higher than standard heparins.

The placebo controlled double blind crossover study was conducted according to the proposals of the EG-GCP-Note

for Guidance. 1216 MS patients were divided into seven randomized groups with a male female ratio of 1:1,7 and a mean age of 29,7 ± 6,2 years.

Table 1: Population Characteristics

Group	sex ratio m / f	mean age years ± 2S	disease duration years ± 2S	Group size n
Whole population	1 / 1.9	31.1 ± 6.22	6.6 ± 1.32	1216
LMWH	1 / 1.7	23.4 ± 4.68	6.8 ± 1.36	323
DEXA METHASON	1 / 2.1	33.2 ± 6.64	7.4 ± 1.48	287
AZA THIOPRIN	1 / 1.9	29.7 ± 5.94	5.9 ± 1.18	302
PLACEBO	1 / 2.0	38.1 ± 7.62	6.3 ± 1,26	304

Acute cases out of all groups	1 / 1.7	31.1 ± 6.22	6.6 ± 1.32	73
LMWH	1 / 1.7	23.4 ± 4.68	6.8 ± 1.36	26
Dexa-methason	1 / 2.1	33.2 ± 6.64	7.4 ± 1.48	23
Azathioprin	1 / 1.9	29.7 ± 5.94	5.9 ± 1.18	24

The control group had a male female ratio of 1:2 and a mean age of 33,2 + 7,4 years. Each group was treated over six months by the same physician with 5 i.U. Fragmin per kg and day with a maximum dose of 300 i.U. Fragmin-D plus 31,2 i.U. Alprostadil per day, dexamethason plus 31,2 i.U. Alprostadil, azathioprine 2,5 mg/kg per day plus 31,2 i.U. Alprostadil or placebo (NaCl 9,9%) plus 31,2 i.U. Alprostadil. The patients received one subcutaneous injection once a day and three times a day the prostavasine intranasal. The maximum dose of 300 i.U. per day for LMWH's was chosen to minimize the risk of insufficient immunocompetent heparin treatment and to avoid high doses of heparin in patients with designated risk factors for bleeding and to reduce the receptor desensitization effect of heparin doses exceeding 5 i.U. per kg and day.

Each patient had a neurologic evaluation done once a week and a Nuclear Magnetic Resonance scan (NMR-scan) once a month. This neurologic examination consisted of a neurologic status (reflexes, visus, muscular tonus, coordination and a dynamometric evaluation of the grasp force (Dynacheque-test)). See table 4.

Concerning this status the criteria were: reduced (-1 point), unchanged (0 points) or improved (+1 point). These data were evaluated in a variance analysis.

Table 4: Variance analysis of the variables: reflexes, visus, muscular tonus, coordination and Dynacheque-test during the different treatment periods. p stands for the significance niveau.

Variables	LMWH n = 323	DEXA n = 287	AZA n = 302	PLACEBO n = 304
reflexes (R)	F:1,856 p:0,000	F:2,364 p:0,069	F:6,473 p:0,051	F:2,751 p:0,141
visus (V)	F:47,99 p:0,000	F:20,69 p:0,701	F:3,630 p:0,865	F:47,08 p:0,965
muscular tonus (M)	F:8,674 p:0,000	F:15,69 p:0,654	F:0,029 p:0,865	F:17,93 p:0,913
coordination (C)	F:3,681 p:0,012	F:91,65 p:0,053	F:4,130 p:0,042	F:11,27 p:0,756
Dynacheque-test(D)	F:74,23 p:0,000	F:1,266 p:0,268	F:1,066 p:0,38	F:0,399 p:0.843

In the NMR-scans number and size of the plaques were documented. After six months the crossover took place so that within 24 months each patient received a six month treatment course of Fragmin-D, dexamethason, azathioprine and placebo. The physicians treating the patients and evaluating their neurological status did not know which therapy the patients received. The medication was prepared as an injectable solution with a volume of 1 ml in ampules carrying a code, the daily injected amount was 1ml. In addition we received blood samples once a week conducting adhesion assays. After the 24 month treatment period the initial LMWH group received a further continuous treatment for 18 months with Fragmin-D.

Results are shown in table 3.

Table 3: NMR-scans of the central nervous system.

Group	Number of Plaques MEAN ± 2S	Size of Plaques mm² ± 2S
WHOLE POPULATION BEFORE TREATMENT n = 1216	35.6 ± 14.2	65.9 ± 26.4
6 months treatment with LMWH	17.2 ± 6.9	31.4 ± 13.2
after 2 ys LMWH n = 323	4.3 ± 1.7	7.8 ± 2.6
DEXAMETHASON 6 mon n = 287	26.7 ± 10.7	49.4 ± 19.8
AZATHIOPRIN 6 mon n = 302	23.4 ± 9.4	37.8 ± 15.1
PLACEBO 6 mon n = 304	32.1 ± 12.8	59.4 ± 23.8

Change of the number and the size of sclerotic plaques in the different groups which are found in the NMR-scans are shown. Comparing the dexamethason and azathioprine group to the LMWH group the NMR controlled reduction of sclerotic patches in the LMWH group (FRAGMIN-D) is significant (p=0,002; n=1216). Compared to other standard drugs such as Dexamethason and Azathioprin the number of negative side effects is negligible. Figure 4 shows the mean therapeutic results graphically.

There were 73 acute cases of MS amongst the group of 1216 patients of example 1; see table 1 and also Fig. 5. These patients had a breething failure and needed a mechanical breething support on admission. They were randomized into three groups and treated either with a LMWH i.e. Fragmin-D, dexamethason or azathioprine. The only improvement

criteria was the possibility to reduce or turn off the mechanical breathing support.

A significant therapeutic improvement in the 73 acute cases could be demonstrated after treatment with LMWH as compared to treatment with dexamethason or azathioprine (p = 0,002; n = 73). Patients treated with Fragmin-D showed the highest amount of reduction of vitally impairing symptoms.

Example 2:
Adhesion assays were performed with blood samples from MS patients treated with low-molecular-weight heparin, dexamethason, azathioprine and a control substance. Adhesion assays were carried out as described above. The results are shown in table 2.

Table 2: Pathologic adhesion pattern of lymphocytes/cell.

Group	HEC	Oligodendrocytes	Schwann´cells
	percent per 100 cells ± 2S		
LMWH n = 323	1.2 ± 0.2	0	0
DEXA METHASON n = 287	45.6 ± 19.2	52.8 ± 21.2	34.7 ± 13.9
AZATHIOPRIN n = 302	97.5 ± 48.8	85,9 ± 45.4	69.4 ± 27.8
PLACEBO n= 304	450,6 ± 246,2	234.6 ± 117.5	237.1 ± 118.6
WHOLE POPULATION n = 1216	148.7 ± 53.6	122.1 ± 61.1	96.4 ± 38.6
Healthy Control n = 200	1.4 ± 0.3	0	0

To determine the anti-inflammatory and anti-activation potential of heparins versus corticosteroids and azathioprine we preincubated Ta_1 and $T11_3$ cells isolated from the MS patients for 60min at 37°C with 0,004 i.U./ml LMWH, with 0,0014 mg/ml dexamethason or with 0,0025mg/ml azathioprine and performed an adhesion assay on pICAMhec expressing HEC, oligodendrocytes and Schwann cells which showed no pathologically increased adhesion in case of LMWH-pretreatment while the pathologic adhesion patterns were not positivly influenced by dexamethason or azathioprine.

Refering to table 2 the in vitro adhesion assays showed pathognomonic patterns of lymphocyte adhesion on in vitro cultivated HEC. According to Woodruff et al. (1980) the physiologic adhesion rate is one lymphocyte per high endothelial cell. We observed similar rates. Putting pathophysiologic conditions into account such as MS the adhesion rate varies from 2 lymphocytes per HEC to 10.000 lymphocytes per HEC depending on the state of disease and activity.

Fig. 7 shows the typical alteration of the adhesion rate in a case of MS. The last attack is already 15 years ago.

Fig. 8 reveals the typical picture of an acute MS attack. By dilution row we managed to observe more than 10.000 adherent lymphocytes per HEC.

The adhesion assay was routinely performed for all patients and we observed that the assay is a highly specific and the most sensitive tool for diagnosis of MS compared to the Nuclear Magnetic Resonance Scan which needs sclerotic patches of at least 5mm diameter in vivo to come

up with a diagnosis. This is due to the fact that the adhesion assay shows changes of the adhesion pattern immediately even when the patient has not got any symptoms.

The results of table 2 show that the adhesion is more efficiently reduced with LMWH compared to dexamethason and azathioprine. Moreover, the rate of adhesion upon heparin treatment is similar to that of healthy control individuals.

Example 3:
There are also other diseases that have been treated with heparins, heparinoids, proteoglycans and LWHM in doubleblind crossover studies. In these studies 174 patients with Alzheimer's disease, 174 with GvH, 174 with leukaemia, 174 with lymphomas, 174 with primary biliary cirrhosis, 173 with rheumatism and 173 patients with neuritides (german: Neuritiden) have been treated with the above-mentioned pharmaceuticals, corticosteroids and placebo.

It turned out that results similar to those obtained by treatment of MS patients with LWHM have been received by treatment of rheumatism, primary biliary cirrhosis (PBC), neuritides if autoagressive (this means if it has an immunological origin), GvH and systemic carcinoma e.g. leukaemia and lymphomas with the same pharmaceuticals.

Most suitable for the treatment of rheumatism, PBC, neuritides and of course MS are pharmaceuticals as Fragmin and Fragmin-D which are both LWHM's. Fragmin-D has 2.500 i.U./ml and Fragmin has 10.000 i.U./ml. Thus, Fragmin has a higher yield than Fragmin-D and can be cheaper in use.

Fragmin-P is also suitable for the treatment of the above-mentioned diseases.

Most suitable for the treatment of GvH and systemic carcinoma e.g. leukaemia and lymphomas is Reviparin. The bone marrow diffusion of Reviparin is much better than that of Fragmin or Fragmin-D. There is a higher concentration of a factor 10^3 of this pharmaceutical in the bone marrow which is a result of the higher diffusion (also a factor of 10^3).

All other LWHM's which can be bought until now vary in concentration up to 20% . Thus, the usage for treatment is much more intensive in time because the correct concentration has to be determined before it can be used to treat the patients.

It turned out that Fragmin and Fragmin-D is also very suitable to treat patients with migraine and hyper-IgE-syndrome.

To treat Crohn's disease a special medication (application) has to be used. In this case pills with a galenic preparation that is soluble in the small intestine is used. Thus, it is possible to treat the disease where it occurs with very few side effects.

What is claimed is:

1. Pharmaceutical for the treatment of inflammatory or immunological diseases
characterized in that the pharmaceutical comprises heparins, heparinoids, proteoglycans or low-molecular-weight heparins or a mixture thereof or a combination of low-molecular-weight heparins and Prostavasin.

2. Pharmaceutical according to claim 1,
characterized in that the pharmaceutical comprises Fragmin-D, Fragmin, Fragmin-P and/or Reviparin.

3. Pharmaceutical according to claim 1 or 2,
characterized in that a dosage between 1 and 40 i.U. per kg and day (for heparins with the exception of LMWH up to 1200 i.U. per kg and day) is used.

4. Pharmaceutical according to claim 1 or 2,
characterized in that a dosage between 1 and 5 i.U. per kg and day is used.

5. Method of treatment of inflammatory or immunological diseases,
characterized in that patients are treated with a pharmaceutical composition comprising heparins, heparinoids, proteoglycans or low-molecular-weight heparins or a mixture thereof or a combination of low-molecular-weight heparins and Prostavasin.

6. Method of treatment according to claim 5,
characterized in that the pharmaceutical composition comprises Fragmin-D, Fragmin, Fragmin-P and/or Reviparin.

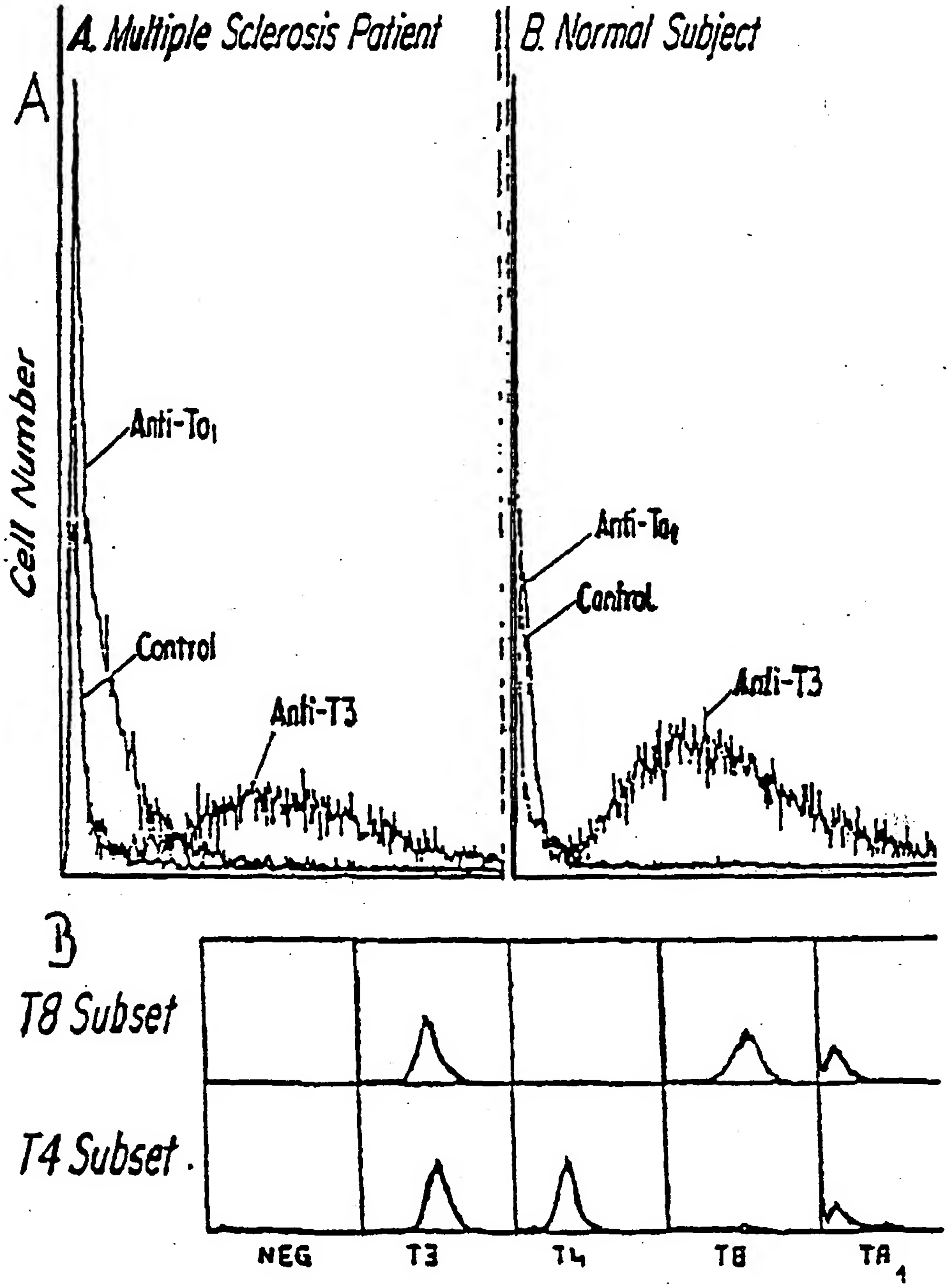
7. Method of treatment according to claim 5 or 6, characterized in that the disease is multiple sclerosis (MS), Ghost versus Host reaction (GvH), primary biliary cirrhosis, post-infarct-syndrome, lupus erythematosus (LE), rheumatism, migraine, hyper-IgE-syndrome, neurotides, Crohn's disease or systemic carcinoma e.g. leukaemia or lymphomas.

8. Method of treatment according to claim 5, 6 or 7, wherein the treatment is directly pointed to the mucosa and/or an injection is performed hypodermicly or intravenously.

9. Method of treatment according to claim 8, characterized in that, a nasally treatment occurs via a spray.

10. Method of treatment according to claim 5, 6 or 7, wherein pills are swallowed which have a galenic preparation being soluble in the small intestine.

Figure 1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Anti-T3
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T3
T4
T8
TA 4

Figure 2

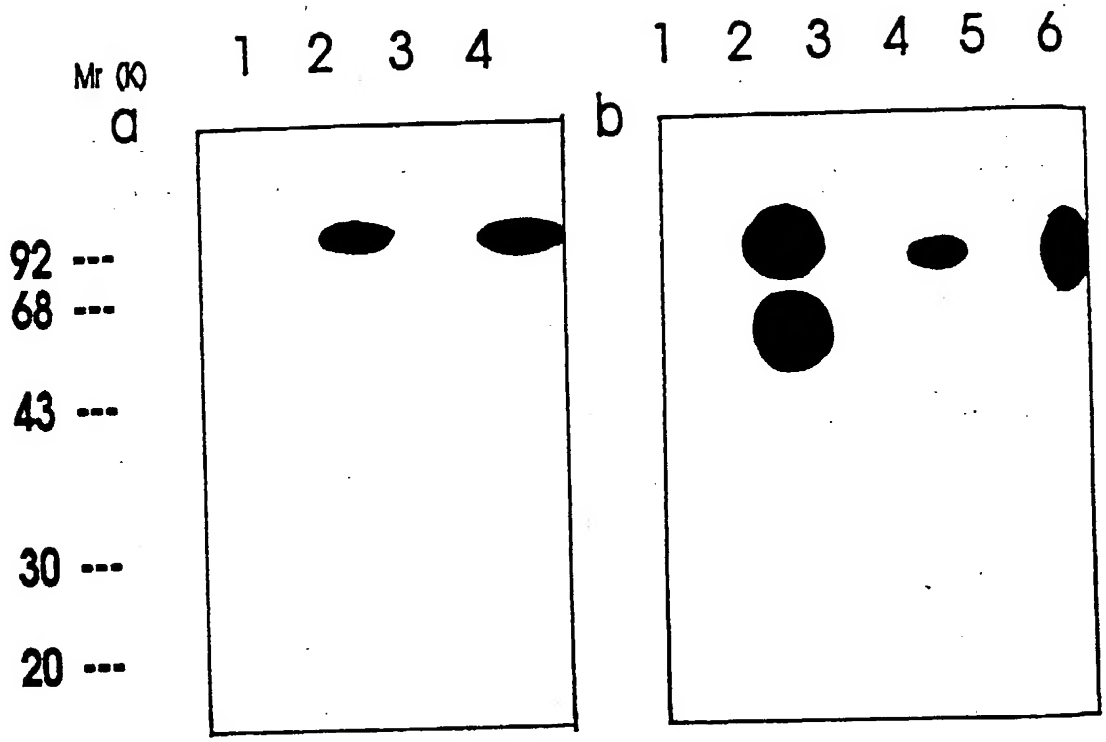


Figure 3

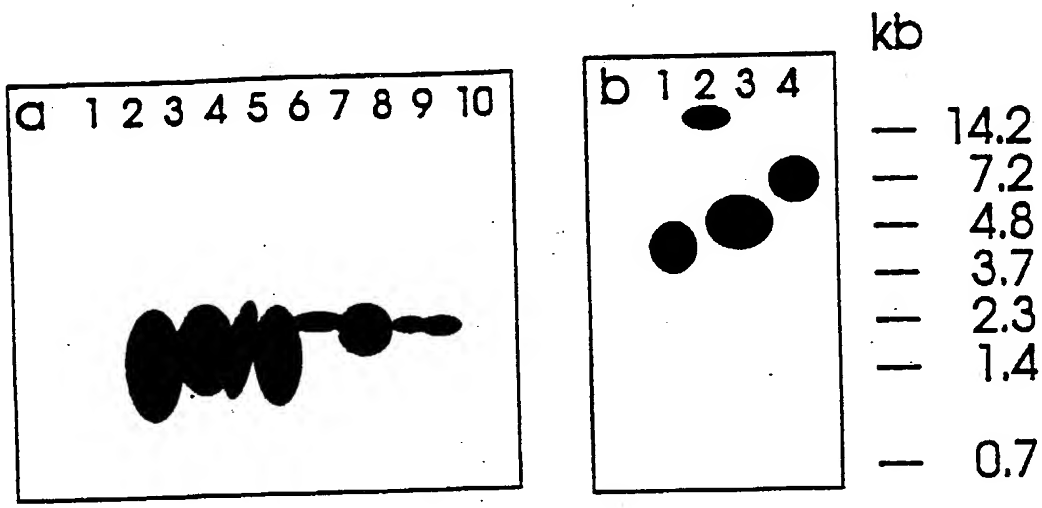


Figure 4

Mean reduction of sclerotic patches and symptom improvement after longterm treatment

Observation period 1985-1991

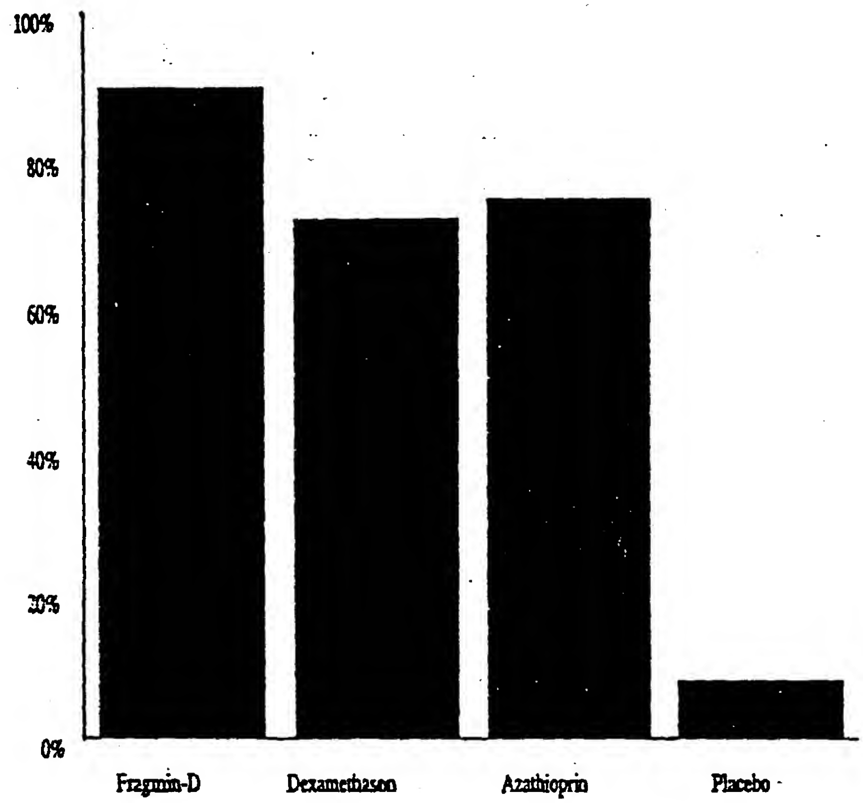
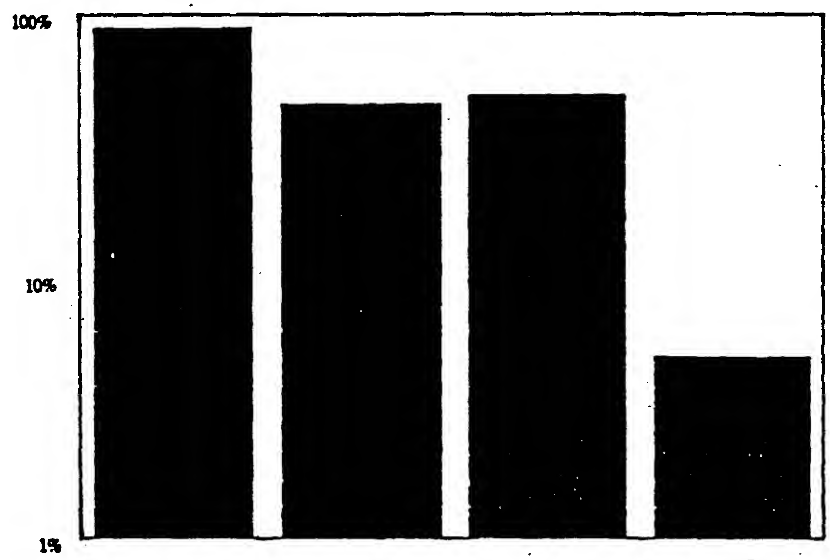


Figure 5

Reduction of vitally impairing symptoms in acute cases i.e. breathing failure within 48h treatment

Observation period 1985-1991



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(-25) (+1) (+11)

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(+51)

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(+91)

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Fig. 6a

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ICAM  -------KVYELSNVQEDSQPMCYSNCPDG-QSTAKTFLTVYWTPERVELAPLPS
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NCAM  PQEFKEGEDAVIVCDVVSSLPP---TIIWKHKGRDVILKKDVRFIVLSNNYLQIR   165
ICAM  WQPV--GKNLTLRCQVEGGAPRANLTVVLLRGEKE--LKREPA-VGEPAEVTTTV
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       ••• ••G• •  C   • L    ••  F •     ••   V   •   •V     •

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      ••   T•VC  DG FP  E• •  • ••• ••       •• S S  •  V••

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Fig. 6b

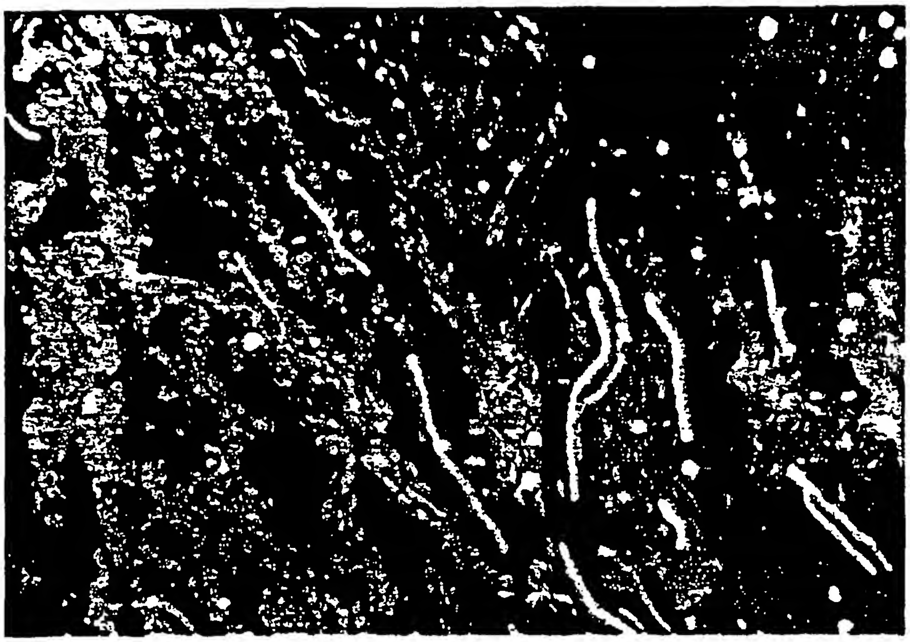

Fig. 7

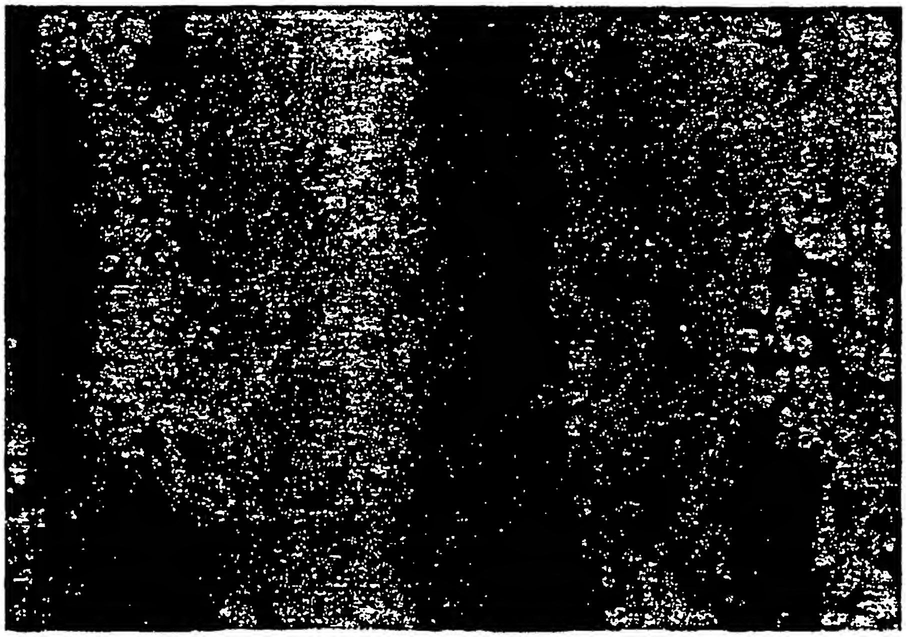

Fig. 8